Synopsis of thesis entitled

Differential role of FSH: A study using Sertoli cells and epididymal cells

submitted by

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for the award of the Ph D degree of the Indian Institute of Science, Bangalore, India

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Cellular differentiation is a complex process during which undifferentiated cells undergo a variety of changes, including both morphological and biochemical changes, enabling the cells to carry out specialized functions The involvement of hormones and growth factors in the process of cellular differentiation is well established For example, it is known that Gonadotropin Releasing Hormone (GnRH) and estradiol 17 β are involved in the differentiation of the uninucleated cytotrophoblast into multinucleated synctiotrophoblasts which are functionally very active cells in the human placenta Similarly, in the male reproductive system, Luteinizing Hormone (LH) induces differentiation of progenitor Leydig cells into adult Leydig cells which are capable of producing testosterone, indispensable for normal spermatogenesis The results of studies presented in this thesis deal with differentiation of Sertoli cells and role of Follicle Stimulating Hormone (FSH) in this process Sertoli cells are an important part of the male reproductive system The male reproductive system essentially consists of a pair of testes, a pair of epididymes along with their ducts and accessory glands Testis is divided into two compartments by the seminiferous tubules Sertoli cells and germ cells lie in the tubular compartment whereas Leydig cells are situated in the interstitial compartment Sertoli cells provides a microenvironment for the differentiating germ cells by forming gap junctions between Sertoli cells and cell to cell interactions between Sertoli cell-Leydig cells, Sertoli cell- Germ cells and Sertoli cell- peritubular cells

The main function of testis is production of germ cells and synthesis and release of the male sex hormone testosterone Testosterone is produced by the Leydig cells, the growth and function of which are regulated by LH The germ cells undergo differentiation into spermatozoa in the testis and through the rete testis they are released into the epididymis In epididymis they undergo the process of maturation and acquire motility and ability to fertilize the egg The Sertoli cells under the influence of FSH synthesize and secrete a variety of factors which influence the function of Leydig cells, peritubular cells and differentiation of germ cells The differentiation of Sertoli cells from immature stage into adult stage is regulated by FSH It is interesting to note that besides stimulation of proliferation of the Sertoli cells, FSH also has a differential action during the two stages of immature (undifferentiated) and adult (differentiated) Sertoli cells

FSH, also known as Follitropin, an anterior pituitary glycoprotein hormone is known to have a specific and selective action on Sertoli cells in males via interaction with its membrane receptor FSH regulates cell proliferation only during the immature stages of Sertoli cells (till day 18 in rats) and beyond this period, the Sertoli cells do not divide even in the presence of FSH Thus during the adult stages, beyond day 20 in rat, FSH regulates the other functions of Sertoli cells which include maintenance of the microenvironment by developing intercellular tight junctions, secretion of growth factors and binding proteins that help in transporting important molecules to the developing sperm and secretion of paracrine factors for inter-cell communication to regulate androgen production Available evidence indicates that in other species including primates, FSH exerts differential action on Sertoli cells Thus, Sertoli cells from immature and adult rats, in which FSH exerts a differential action, obviously can serve a very suitable model to understand the basis for the differential action **Accordingly the objective of the present study is to obtain an insight into the possible mechanism by which FSH exerts the differential action on Sertoli cells from immature and adult rats. An additional objective is to employ the immature rat cauda cells in which we have been able to demonstrate the presence of the FSH receptors (FSH-R) serendipitously for the first time**

and understand the possible significance of the FSH-R in cauda region of epididymis. Recent studies using FSH-R knockout and FSH-β subunit knockout males have demonstrated the importance of FSH and its receptor signaling system in maintaining testicular homeostasis for optimal sperm production and development Accordingly the experiments designed in the present study are based on the hypothesis, that changes in FSH receptor expression, signaling mechanism and differential gene expression may be responsible for the differential action of FSH on immature and adult rat Sertoli cells

In **Chapter 1** a critical account of the available literature on male reproduction and its hormonal control is presented Emphasis is placed on the importance of Sertoli cells, their structure, hormonal control of growth and function and cell-cell interaction The role of FSH, testosterone and thyroid hormone in the regulation of Sertoli cell proliferation and differentiation is discussed This chapter also provides available information on the differential action of FSH during the two stages of Sertoli cell development and the markers available to evaluate the differential action The possible mechanism involved in the differential action is hypothesized to be at the level of differences in the FSH-R, signaling molecules or the expression of the downstream genes

Chapter 2 provides a detailed account of the approaches employed to understand the differential action of FSH on Sertoli cells Thus the methods to isolate Sertoli cells from immature and adult rats and their characterization are presented Since the approach also includes the evaluation of effects of addition of FSH as well as deprivation of FSH under *in vitro* and *in vivo* conditions respectively, methods for culture of Sertoli cells and monitoring their proliferation and viability are presented As the Sertoli cells are known to be responsive to FSH as well as testosterone, any possible contamination of oFSH preparation used to study the effect of addition or deprival by using antiserum raised against oFSH, will make it difficult to draw conclusions with certainty Accordingly, this chapter also provides a detailed account of the demonstration of presence of trace amounts of LH even in highly purified

preparation of oFSH and methods to remove the contamination and analyze the specificity of the FSH antiserum used in the studies The use of a specific antibody against FSH to neutralize the endogenous FSH in rats permits selective neutralization for a desired duration and thus mimics situation of a conditional knock out Ovine FSH was processed by passing through a LH antibody affinity column to get rid of the LH contamination present in the oFSH preparation The purity of FSH was established by adult Leydig cell testosterone production assay The absence of LH antibodies in the FSH antiserum was ascertained by absence of binding of the antiserum to ^{125}I-hCG and absence of effect on the nocturnal surge of serum testosterone levels in the adult male bonnet monkeys used for immunization against oFSH The protocols employed for these techniques are described in **Chapter 2** The other cell isolation protocols include the methods for isolating Leydig cells and cells from cauda epididymis of rats The details of procedures employed for isolation of RNA, RT-PCR, DD-RT-PCR, northern blot analysis and micro array analysis are also described in this chapter The procedures employed to carry out western blot and to demonstrate binding of radioactively labeled ligand to receptors or to the antibody are also described in this chapter The treatment schedules, quantity of hormones or volume of antiserum given to immature and adult rats have been described in this chapter The chapter also includes histological and immunohistochemical protocols employed during the study to evaluate the effects of neutralization of FSH or LH and for demonstration of FSH receptors in different regions of epididymis The procedures for measurement of cAMP stimulation and BrdU incorporation are discussed The details of steroid antisera employed, procedures followed for RIA are also described in this chapter

The results obtained in the present study are presented in four chapters (**Chapter 3- 6) Chapter 3** provides results of studies carried out to validate the model system employed in the present study Following isolation of Sertoli cells from immature and adult rats the cells were characterized as Sertoli cells by RT-PCR analysis using markers specific for Sertoli cells The cells were observed to be positive for androgen binding protein (ABP), transferrin and FSH-R as assessed by RT-PCR analysis The absence of

Leydig cell contamination was confirmed by ascertaining the absence of signal for the Leydig cell specific parameter, namely LH receptor, by RT-PCR analysis The results of experiments carried out to establish the purity of oFSH and the absence of LH antibodies in the FSH antiserum employed are described in this chapter The bioneutralizing capability of FSH a/s was assessed by analyzing the effect on the weight of testis in immature rat

Once the system was validated, Sertoli cells isolated from immature and adult rats were compared for expression of parameters indicative of either proliferation or function The level of expression of Cyclin A2 and proliferating cell nuclear antigen (PCNA) were monitored by RT-PCR for assessing cell proliferation, while the level of expression of ABP and transferrin were considered for the functional parameters It was observed that the level of expression of the proliferative parameters is more in the immature Sertoli cells whereas the expression of the functional parameters was more in the adult rat Sertoli cells The role of FSH in the proliferation of immature Sertoli cells was established following neutralization of endogenous FSH by injecting antiserum to FSH and assessing the effect on BrdU incorporation by the Sertoli cells Compared to the normal monkey serum (NMS) treated animals which served as controls, the incorporation of BrdU by the Sertoli cells from FSH a/s treated immature rat Sertoli cells was significantly decreased The effect of FSH deprival on the cell cycle markers in immature rat Sertoli cells was analyzed using cyclin and cyclin dependent kinase inhibitors as cell proliferation markers by RT-PCR and western blot analysis The results established the importance of FSH-R for regulation of proliferation of immature Sertoli cells A similar study was not carried out in the adult rat Sertoli cells as it is well established that even following addition of FSH, these cells do not proliferate The regulation of functional parameters by FSH was studied by monitoring the effect of deprival and addition of FSH on transferrin The role of FSH in regulation of Sertoli cell function was analyzed both under *in vitro* and *in vivo* conditions The results of this study revealed that the increase observed following addition of FSH in the level of expression of transferrin was more in the adult Sertoli cells both under *in vivo* and *in vitro* conditions The results of these experiments are discussed in **chapter 4** In

order to understand the mechanism of the differential action of FSH during the two stages of Sertoli cells, we hypothesized that the effect could be at the level of receptor, signaling pathway and the expression and regulation of the downstream genes Initially the immature and adult rat Sertoli cells were compared for the level of expression of FSH-R which was less in the adult rat Sertoli cells as analyzed by RT-PCR and northern blot analysis It was also observed that the expression of both the transcripts of FSH-R i e 4 5 Kb and 2 6 Kb was less in the Sertoli cells from adult rats The regulation of FSH-R by FSH was observed only in the immature rat Sertoli cells and not in the adult rat Sertoli cells suggesting that the difference in the response of the immature and adult rat Sertoli cells to FSH could be at the receptor level One of the important signaling molecules by the cAMP pathway is Protein Kinase Inhibitor (PKI) which is responsible for the inhibition of the activity of Protein kinase A (PKA) PKA is important for the phosphorlyation of the downstream genes involved in the cell cycle and thus is important for the proliferation of Sertoli cells The level of expression of PKI was found to be higher in the adult rat Sertoli cells, which was further elevated upon stimulation with FSH The mobilization of extracellular calcium following addition of FSH was observed only in the immature rat Sertoli cells These results suggest that differences exist in the signaling pathway operative in the two stages of the rat Sertoli cells and these differences could be responsible for the differential action of FSH during the two stages of Sertoli cells Our studies also demonstrated that IL 6 which is an important gene involved in the proliferation of the endothelial and myeloma cells is highly expressed only in the immature rat Sertoli cells Addition of FSH increased the levels of IL 6 only in the immature rat Sertoli cells In order to understand the differential action of FSH on genes which may be involved in regulation of proliferation, we carried out a DD-RT-PCR analysis using the RNA isolated from the FSH a/s and NMS treated immature rat Sertoli cells The DD-RT-PCR analysis revealed the down-regulation of a gene, the sequence of which shared 90% homology with the Eker rat intracisternal A particle element (IAP) IAP belongs to the family of endogenous retroviruses that have the capability of integrating in the host genome and thus being regulated along with those genes In the immature rat Sertoli cells, the regulation of IAP was also confirmed using the *in vitro* model

system However, there was no difference in the level of expression of IAP in the immature and adult rat Sertoli cells Further more, by employing both the *in vivo* as well as the *in vitro* model system we also observed that FSH did not regulate the expression of IAP in the adult rat Sertoli cells This suggests that IAP could have integrated into a gene which is either directly or indirectly regulated by FSH only during the immature stage The results of these studies are also presented in **chapter 4.**

It is well established that FSH is important for the proliferation of Sertoli cells during the early neonatal period However, during the post pubertal stages, even in the presence of FSH the Sertoli cells do not proliferate This suggests the possible involvement of other factors, which in close association with FSH induce the differentiation of the Sertoli cells Studies have demonstrated that thyroid hormone is responsible for the inhibition of Sertoli cell proliferation There are two model systems available to study the role of thyroid hormone in regulation of Sertoli cell differentiation One of the model systems involves induction of neonatal hyperthyroidism by administration of thyroxine to immature rats The second model system is to induce hypothyroidism in neonatal rats by using a reversible goitrogen n-propyl thiouracil (PTU) in the drinking water to the nursing mothers In our studies presented in **chapter 5** we have employed the hyperthyroid rat model system to study the role of thyroid hormone in the growth and differentiation of the immature rat Sertoli cells Following administration of thyroxine to immature rats, there was a significant decrease in the BrdU incorporation by the Sertoli cells from treated rats by day 13, indicating arrest in the proliferation It is pertinent to recall here that proliferation was also decreased following deprival of endogenous FSH in immature rats by administration of FSH a/s Thus, both FSH deprival and hyperthyroidism resulted in the inhibition of Sertoli cell proliferation However, while the level of expression of FSH-R increased following FSH deprival, a decrease in the level of FSH-R expression was observed following thyroxine treatment It is known that FSH levels are unaltered following hyperthyroidism and thus the mechanism of inhibition of proliferation appears to be different in the two model systems It may be recalled that we had observed that following FSH deprival there is a decrease

in the level of expression of cell cycle markers such as cyclin and PCNA, without a concomitant increase in the functional parameters This suggests that deprival of FSH alone is not adequate to induce differentiation of Sertoli cells Considering this important difference in the inhibition of proliferation by FSH deprival and thyroxine treatment, it was of interest to investigate gene expression profile under hyperthyroid conditions in immature rat Sertoli cells Accordingly micro array analysis was carried using Rat Oligo array having 5,600 genes The results of micro array analysis revealed the up-regulation of known Sertoli cell functional markers such as ABP, transferrin, protein kinase and their inhibitors, clusterin, and cystatin-TE following hyperthyroidism However cell growth promoters like early growth response protein, connective tissue growth factor and collagen XII alpha 1 were down-regulated following neonatal hyperthyroidism The results of micro array analysis support the hypothesis that thyroid hormone inhibits Sertoli cell proliferation and induces differentiation In order to test this, we monitored the expression profile of these genes in the immature and adult rat Sertoli cells which represent the proliferating and differentiated cells respectively RT-PCR analysis revealed that the pattern of expression for the genes selected in micro array analysis was same in the immature and adult rat Sertoli cells as seen in the euthyroid and hyperthyroid rat Sertoli cells This suggests that the Sertoli cells from immature rats have differentiated following exposure to thyroid hormone The expression profile of the proliferation and functional parameters monitored in the hyperthyroid rat model system were also analyzed following FSH deprival in immature rat Sertoli cells However, in this model the pattern of gene expression was not the same as observed following hyperthyroidism While the level of proliferation promoters such as expression of early growth response protein and collagen XII alpha 1 was unchanged, the level of connective tissue growth factor was up-regulated In contrast, the functional marker clusterin was unchanged and cystatin TE was down-regulated following FSH deprival This supports the hypothesis that there is a difference in the molecular mechanism responsible for the decrease in the proliferation observed in the immature Sertoli cell following FSH deprival and following hyperthyroidism Thus, while FSH deprival by administration of FSH a/s results only in arrest of proliferation, hyperthyroidism not only inhibits

proliferation but also induces differentiation These results presented in **chapter 5** suggest that exposure of Sertoli cells to thyroid hormone during immature stage is responsible for the functional maturity of the cells even before its normal period Thus, there is a balance between the mitogenic effects of FSH and the inhibitory effects of thyroid hormone which is responsible for the cessation of proliferation of immature Sertoli cells and induction of their differentiation

In a study which involved elucidating the mechanism of differential action of FSH on Sertoli cells by employing specific antiserum to neutralize endogenous FSH in rats, we observed significant decrease in the weight of the epididymis The effect of FSH deprival on epididymis was also confirmed by the changes in the histology of the different regions of epididymis Although epididymis is an androgen dependent tissue, the possibility of the observed effects in epididymis being due to deprival of testosterone was ruled out by the fact that there was no change in the level of serum testosterone following administration of FSH antiserum It is pertinent to note here that this antiserum was characterized for the absence of antibodies to LH which could have decreased the levels of testosterone resulting in observed changes in the weight and histology of epididymis This suggested the possibility that epididymis could be a direct target for FSH action The results of this study are presented in **chapter 6** where we have demonstrated the presence of functional receptor for FSH in cauda region of epididymis and evaluated its significance Initially we analyzed the expression of FSH-R in the different regions of rat epididymis by RT-PCR analysis using specific primers for FSH-R RT-PCR analysis gave an amplification product of expected size only from the cauda region of rat epididymis which corresponded to the amplification product seen in the Sertoli cells The identity of the amplified product as FSH-R was ascertained by sequencing Rat Sertoli cells are known to express FSH-R of the sizes corresponding to 2 6 kb and 4 5 kb as analyzed by northern blot analysis However, we could demonstrate presence of only the 2 6 kb transcript in cauda The failure to detect the 4 5 kb transcript may be due to the extremely low levels of expression Western blot analysis, carried out using the solubilized membrane preparation from rat cauda and Sertoli

cells, revealed a band of 75 kDa indicating that the protein for FSH-R is also expressed in the rat cauda By RT-PCR, northern blot and western blot analysis it was observed that the level of FSH-R expression was much low in the cauda compared to Sertoli cells The expression of FSH-R in cauda was also confirmed by immunohistochemical analysis The interaction of FSH with its receptor in cauda was analyzed by carrying out the ^{125}I-FSH binding assay Specific binding of ^{125}I-FSH only by the cauda extract from 10-day and 80-day old rat was observed although binding was much lower with cauda extract from 80-day old rat The functional significance of the expression of FSH-R in the cauda was further evaluated by monitoring the stimulation of cAMP production by FSH in cauda cells Following addition of oFSH, a concentration dependent increase in cAMP levels in cauda from both immature and adult rats was observed although the fold stimulation was much lower than seen with Sertoli cell which was included as positive control Since it is known that FSH stimulates proliferation of Sertoli cells, the possibility of such an effect by FSH in cauda cells was also examined BrdU incorporation analysis revealed that proliferation was high in the 10-day old rat cauda cells compared to the 80-day old rat cauda cells This was also supported by the fact that the level of expression of the proliferating cell nuclear antigen (PCNA) was quite low in adult rat cauda compared to the immature rat cauda cells Following addition of oFSH, a concentration dependent increase in the proliferation of 10-day-old rat cauda cells was observed However, no stimulation in proliferation of cauda cells from 80-day old rat was observed even with 1μg/ mL of oFSH It is known that the level of expression of FSH-R varies with age in rat Sertoli cells Although there is no discernable change in level of expression of message for FSH-R with age in cauda, it is possible that FSH stimulates proliferation in caudal cell only from immature rat, indicating that in the adult rat cauda, FSH may have a different function Neutralization of endogenous FSH resulted in the decreased level of PCNA protein in the immature cauda, thus validating the importance of FSH in the proliferation of cauda cells As it is well known that epididymis is an androgen regulated tissue, we evaluated the importance of androgens in the proliferation of immature rat cauda cells Analysis for expression of mRNA for androgen receptor in the immature and

adult rat cauda cells by RT-PCR analysis revealed that the expression of was quite low in the 10-day old rat cauda cells compared to the 80-day old rat cauda cells No stimulation in BrdU incorporation was observed following addition of testosterone which establishes the importance of FSH in cauda cells from immature rats In addition, hCG had no effect on proliferation of caudal cells which establishes the specificity of action of FSH These results on the specific role of FSH in the proliferation of immature rat cauda are described in **chapter 6.**

Chapter 7 provides the general discussion of the results obtained in our studies using the two model system of Sertoli cells and cauda to understand the differential role of FSH

In conclusion this study provides evidence that the differential response of Sertoli cells to FSH during immature and adult stages is due to differential regulation of FSH-R, difference in signaling mechanism and possibly lack of regulation of IAP in adult Our results have shown that a balance between the inhibitory effects of thyroid hormone and mitogenic effects of FSH on Sertoli cell proliferation during the immature stages play a crucial role in regulating the cessation of Sertoli cell proliferation and initiation of their differentiation Additional evidence for this differential effect of FSH during different stages of differentiation of cells is provided by the rat cauda model system in which for the first time we demonstrated the presence of functional FSH receptors